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14. ABSTRACT We have demonstrated that certain transduction peptides such as 12 lysines and 12 arginines can facilitate internalization into breast tumor lines with higher efficiency than smaller polymers of cationic amino acids. In addition, we have demonstrated that PTD-Sma34 worked in conjunction with Ad.TRAIL gene transfer to induce breast tumor apoptosis. We also have demonstrated that membrane bound TRAIL worked more effectively than soluble (secreted) TRAIL to induce breast cancer apoptosis. Moreover, we have demonstrated that co-administration of Ad.TRAIL with PTD-Smac34 resulted in not only enhanced adenoviral transduction of the tumors, but resulted in a stronger apoptotic effect. Finally, we have initiated studies to identify breast cancer specific tumor lines by screening a peptide phage display library both in cell culture as well as in nude mice bearing xenografts. Initial results in prostate tumors has demonstrated the feasibility of this approach and suggests breast tumor specific internalization peptides can be identified.					
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Introduction

In the United States, breast cancer is the leading cause of death for women between the ages of 45 and 55. As the most common malignancy facing women, during 2001 it was estimated that 192,000 women were diagnosed with breast cancer and 40,200 died from the disease. Current management approaches for breast cancer vary based on stage, however therapeutic approaches can include various combinations of surgery, radiation, chemotherapy and hormone therapy. Despite these approaches, the significant mortality from breast cancer has called attention to additional therapies which target dysregulated pathways present in the tumor cells. Alterations in estrogen receptor-mediated signaling, mutations in p53, C-erbB2 overexpression, upregulated p-glycoprotein (gp170) and dysfunction of apoptotic pathways have been associated with poor prognosis in breast cancer. Gene therapy approaches have attempted to target these anomalies present in malignant tissue, however, the efficiency of gene transfer has proven to be the rate-limiting step. Thus there is a need for more efficient systems for intra-cellular delivery of apoptotic or tumor suppressor proteins. One approach for the efficient intra-cellular delivery of proteins involves the use of protein transduction. Amino acid sequences within HIV Tat and *Drosophila* Antennapedia (Antp) proteins, termed PTDs for protein transduction domains, have been shown to facilitate efficient receptor and energy independent internalization of large protein complexes into a wide variety of cell types in culture and *in vivo*. Recently, we have identified a class of cationic peptides similar to HIV TAT and Antp PTD, rich in arginine and lysine, able to facilitate internalization of marker protein complexes into a wide variety of cell types including breast tumor cell lines. In preliminary experiments, we have demonstrated that certain transduction peptides can facilitate internalization into breast tumor lines 5-fold more efficiently than the HIV Tat PTD. We also have previously demonstrated the feasibility of using PTDs for the treatment of cancer in murine tumor models, where a specific cationic peptide was able to efficiently transduce and kill tumor cells following intra-tumoral injection. Intra-tumoral injection of a mitochondrial disruption peptide, KLAK, fused to a cationic transduction domain resulted in significant murine tumor apoptosis and complete tumor regression. *In vitro*, a peptide derived from the C-terminal negative regulatory domain of p53 which leads to stabilization of wild-type and certain mutants of p53, appears to potentiate the apoptotic effects of etoposide or TRAIL in the MCF-7 and ZR75-1 breast cancer lines following PTD-mediated internalization. Similar effects have been observed in these breast cancer lines when using PTD-mediated delivery of NF- κ B inhibitory peptides or peptides derived from the amino terminus of Smac, a protein able to block the anti-apoptotic effect of IAPs (Inhibitors of Apoptosis). The activity of these peptide cargoes *in vitro* against breast cancer cells, coupled with the ability of PTDs to deliver their cargoes with high efficiency to cells *in vivo* holds the potential for generating novel therapies that bypass the limitations of conventional approaches. In addition to the cationic, non-specific transduction peptides, we also have developed a method for screening for tissue-targeted transduction peptides using an M13 peptide phage display library. Using this method we have identified peptides able to transduce human synovial fibroblasts and airway epithelial cells, as well as one peptide able to target prostate tumor lines specifically. Thus the overall goal of the proposal is to optimize and utilize peptide transduction for efficient delivery of therapeutic peptides and eventually proteins into breast cancer cells. This approach could be used for treatment of not only localized tumor by direct injection into the breast, but also could be used for treatment of metastatic disease. The successful completion of the proposed studies should lead to identification of the optimal transduction peptides for internalization of a variety of therapeutic agents, including peptides, proteins and drugs into breast cancer cells that could be used clinically.

Research Progress

Objective 1: *To identify the optimal cationic transduction domain for breast cancer cells as well as identify breast specific transduction domains.*

Task 1. A panel of peptides, 4 to 12 amino acids in length and enriched for arginines and lysines will be screened for their ability to transduce two different breast lines, MCF-7 and ZR75-1. The screening will be performed using biotinylated peptides coupled to two different marker complexes, avidin- β -Gal and avidin-488. (Months 1-6, Year 1)

Progress: As outlined in the previous progress report, we have previously screened a panel of arginine and lysine rich transduction peptides for transduction of MCF-7 and ZR75-1 cells. The results showed that longer poly-cationic peptides are more effective for transduction of the breast tumor cell lines than shorter peptides, similar to that observed for T cells where 12 R and 12K are more effective than 8R and 8K. We have synthesized 12K and 12R-KLAK and SMAC34 peptides for testing for delivery to and induction of apoptosis in human breast cancer cells.

Task 2. The four best peptides for transduction of breast cancer cells will be characterized for intracellular localization by confocal microscopy following internalization by conjugation to avidin-488. The HIV Tat and PTD-5 peptides will be used as positive controls. (Months 3-9, Year 1)

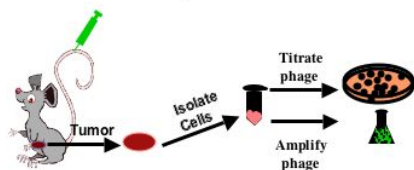
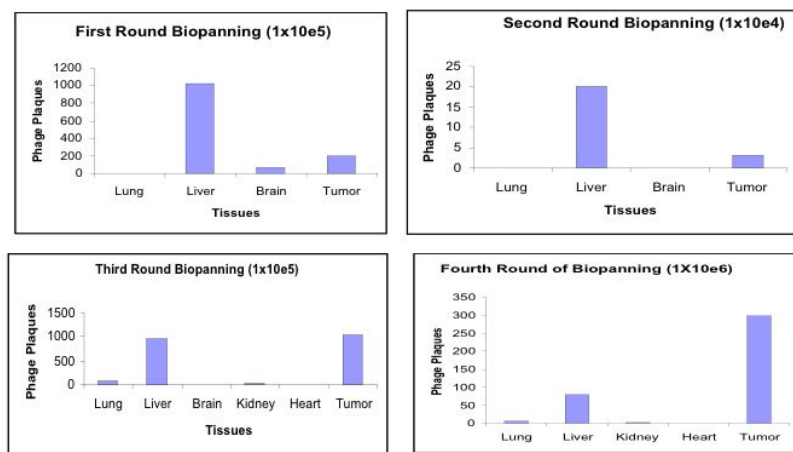
Progress: In preliminary experiments, the 12K and 12R peptides are found to present in both the cytoplasm and nucleus of the cell with more of the 12K in the nucleus.

Task 3. The optimal peptides (maximum of 2), based on their ability to deliver the β -gal and 488 marker complex to the cytoplasmic and/or nucleus of the different breast cancer lines in culture will be examined for ability to transduce breast cancer cells *in vivo*. Nude mice will be inoculated subcutaneously with MCF-7 and ZR75-1 cells. When the tumors reach palpable size, the peptide-avidin- β -gal complexes will be injected intra-tumorally and the extent of transduction examined three hours post-injection by X-gal staining of tumor sections. The HIV and PTD-5 peptides will be used as a positive control. 3 mice will be used per treatment group. However, different doses of peptide-marker conjugates may have to be tested. (Months 6-12, Year 1)

Progress: These experiments are being performed with the 12R and 12K peptide.

Task 4. An M13 peptide phage display library will be used for screening for novel transduction peptides able to facilitate internalization into breast cancer lines in culture. The screen will be performed on MCF-7 and ZR75-1 cells with three rounds of screening. The phage that are isolated will be panned against HeLa cells to eliminate any non-specific internalizing peptides. (Months 1-12, Year 1).

Progress: To screen for peptides able to facilitate tumor internalization, an M13 phage 12 amino acid control peptide display library (New England Biolabs, Beverly, MA) was used as schematically presented below. Briefly, a nude mouse bearing a human tumor line was tail vein injected with 1×10^{12} phage in 0.5 ml TBS, from M13 library. The injected mouse was then sacrificed 3-6 hours later and the mouse lung, spleen, heart, kidney, liver, brain and implanted tumor were surgically isolated. The tissues were washed extensively (20 times) with pH 7.4 Tris-NaCl buffer, the cells were isolated by homogenization and



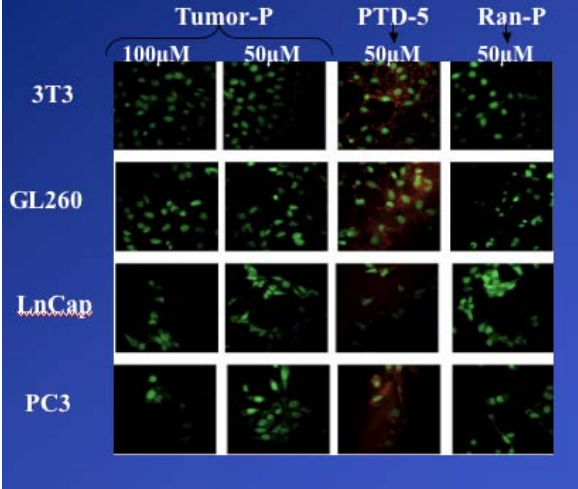
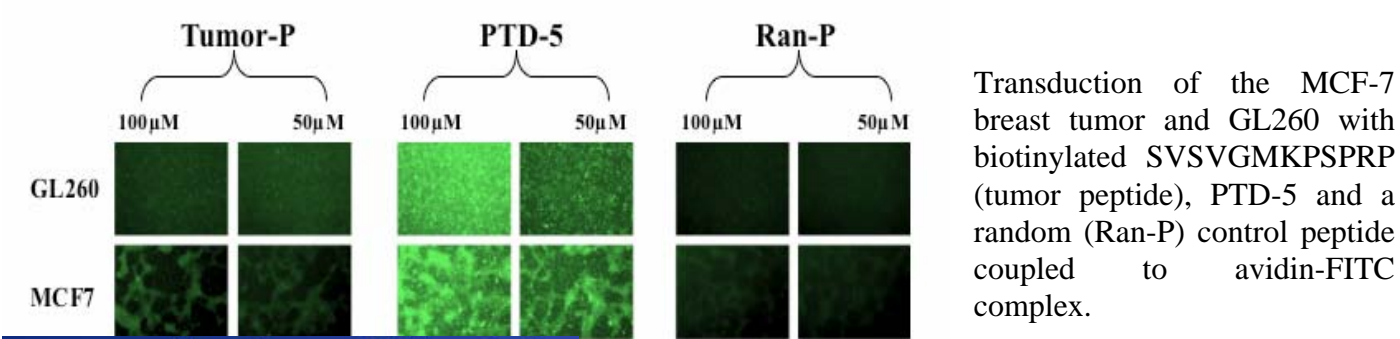
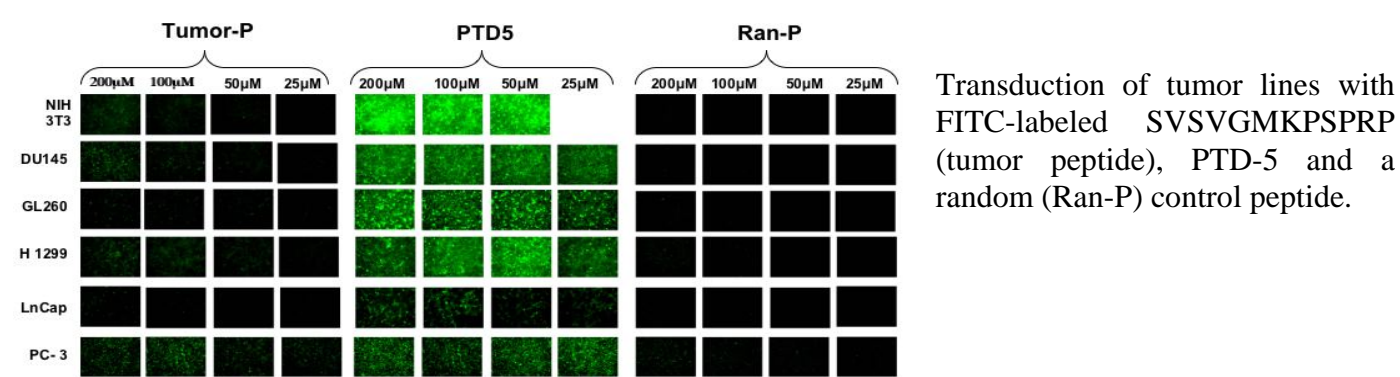
trypsinization and lysed by three consecutive rounds of freeze thaw. The cell lysate was centrifuged and the supernatant removed and saved for phage titration and amplification. We have performed this screen on a human breast tumor line (MCF-7), a human glioma cell line (U87) and a prostate line (LNCaP). The breast and prostate tumor screens are in their 4th rounds where as the human glioma screen has been completed. The data presented below for the human glioma tumor shows that there is significant enrichment in the phage found internalized into the tumor following four rounds of screening. Sequencing of the phage form the fourth round of biopanning had identified a single peptide in over 90% of the phage isolated

(SVSVGMKPSRP). This result suggests that the biopanning approach for internalizing, tumor specific PTDs is working as designed. Interestingly, the peptide identified by the in vivo screen is the same as the peptide identified from an earlier screen on prostate tumor cells in culture. This screen was done on three different cell lines, LnCap, PC-3 and DU145, sequentially to try to insure that the peptides identified were prostate tumor specific, not prostate cell line specific. This fact that we identified the same peptide in both in vivo and in cell culture screens suggests that the same peptide able to be transduce prostate tumor lines in culture also is able to transduce at least one glioma line and possibly other tumor cells in vivo. As outlined below, we have begun to analysis the ability of the identified peptide to transduce a variety of tumor types in culture including breast tumor cells.

Task 5. The ability of the breast screened transduction peptides to facilitate internalization of avidin- β -gal and avidin-488 into breast cancer lines will be examined. In addition, the specificity of transduction will be evaluated by examining transduction of HeLa, Saos-2 and LNCaP cells. (Months 1-6, Year 2)

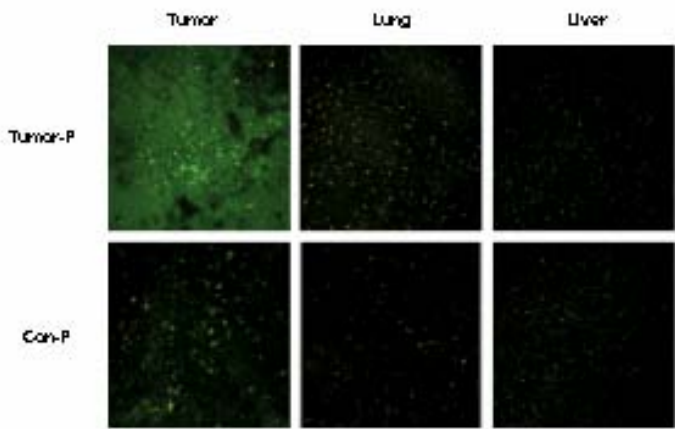
Progress: We have now begun to analyze the uptake of the identified peptide SVSVGMKPSRP into a panel of tumor cells. Although this work is still in progress, it appears as if the phage carrying the SVSVGMKPSRP peptide as well as labeled peptide is not internalized into the cell, but instead interacts extremely well with the membrane of a panel of different tumor cells. This conclusion is based on the

results shown below where analysis by light microscopy shows stronger binding of the FITC labeled SVSVGMKPSRP (tumor peptide) to a panel of a variety of tumor types including breast tumor cells. However, when the analysis was performed using confocal analysis, no labeled tumor peptide was observed inside the cell in contrast to the general transduction peptide (PTD-5). Given that the screening approach used to identify the tumor peptide was designed to identify internalizing peptides, this result is surprising. However, as outlined in a subsequent section, the tumor peptide appears able to home to at least to different types of tumors following IP or IV injection, suggesting that it might be highly useful for diagnostic and therapeutic purposes, to deliver therapeutic agents to the tumor (see below)



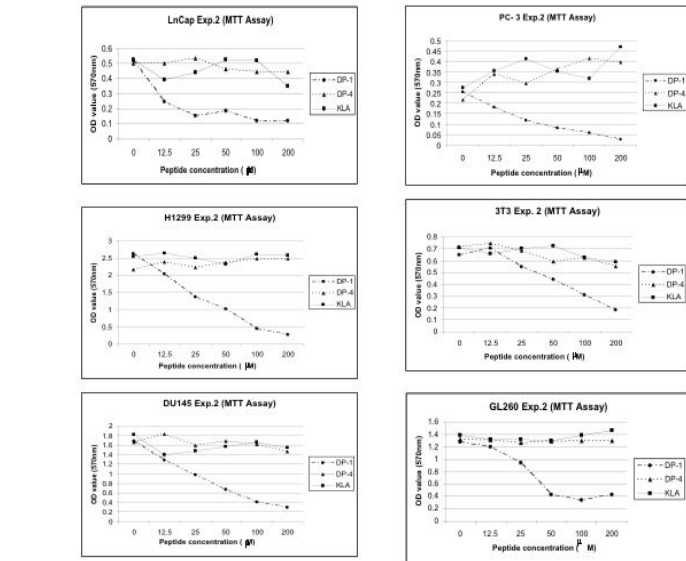
Task 6. The identified breast targeted transduction peptides (maximum of 2) will be examined for their ability to deliver marker complexes into breast cancers in nude mouse xenograft models following intra-tumoral (local) as well as intra-peritoneal (systemic) administration. Nude mice will be inoculated subcutaneously with MCF-7 cells. When the tumors reach palpable size, the peptide-avidin- β -gal complexes will be injected intra-tumorally as well as intravenously and the extent of transduction examined three hours post-injection for the intra-tumoral injection and 6 hours post-injection for the intravenous injection by X-gal staining of tumor sections and by quantitating β -gal activity in tumor lysates. The TAT PTD and PTD-5 peptides will be used as a positive control. 3 mice will be used per treatment group. However, different doses of peptide-marker conjugates may have to be tested. (Months 6-12, Year 2).

Progress: We have begun analyzing the specific SVSVGMKPSRP (tumor peptide) labeled with FITC for analysis of tumor targeting in vivo. In the first set of experiments, SCID mice were inoculated with the GL-260 tumor line. 2 weeks post-inoculation, once the tumor reached a palpable size, the peptide was injected



Analysis of localization of the tumor peptide-FITC 3 hours following i.p. injection into SCID mice with palpable GL-260 tumors.

either i.v. or i.p. and the tumor as well as other tissues isolated 3 hours post-injection for analysis the presence of the labeled peptide. A representative example of the results is shown. All of the labeled tumor peptide was found associated with the tumor xenograft three hours post-injection whereas no labeled peptide was found in liver and spleen. This in is contrast to results using a cationic transduction peptide where extensive uptake is seen in the liver with some transduction in the lung (data not shown). The results are also in contrast to the control labeled peptide that was not found in the tumor or in lung or liver tissue. A similar targeting was observed with the tumor peptide in the PC-3 xenograft model and we are now analyzing the extent of tumor transduction using xeongraft models of breast and head and neck cancers. Thus these results clearly show that ability of the identified peptide to rapidly and efficiently target at least two different types of tumor xenografts.



Objective 2: To examine the ability of peptide mediated transduction of specific agents to regulate breast cancer cell growth and apoptosis.

Task 1. The ability of the 2 optimal cationic and the 2 optimal breast targeted transduction peptides to deliver three different potentially therapeutic peptides will be tested. The three peptides to be tested include one derived from the amino

terminus of Smac, a pro-apoptotic protein, a c-terminal p53 peptide able to activate the transcriptional activity of wildtype p53 as well as certain p53 mutants, a peptide able to block NF-kB activation (NBD), and a general pro-apoptotic factor able to disrupt mitochondria. PTD-5 fused to the Smac, p53, NBD and KLAK peptides will be used as positive controls. The peptide fusions will be examined for ability to inhibit viability or proliferation of breast cancer cells in culture. Increasing concentrations of the different peptide fusions will be added to the media and cell viability measured at different time points by MTT and by FACS analysis following PI and annexin V staining. To examine the tissue specificity of the observed effects, the activity of the peptides will be compared in HeLa, LNCaP and Saos-2 cells. (Months 1-12, Year 2)

Progress: We have fused the identified SVSVGMPKSPRP peptide to the KLAK peptide (DP-4), non-specific inducer of apoptosis through disruption of mitochondria and tested it for induction of tumor apoptosis. As a control, PTD-5 coupled to KLAK (DP-1) was used which is a highly efficient, non cell type specific inducer of apoptosis. As shown below, DP-1 was able to induce apoptosis in all the treated cells whereas DP-4 did not induce apoptosis, even at the highest dose. Since we initially assumed that the SVSVGMPKSPRP peptide was able to be internalized into a variety of human tumor cells, this lack of killing by DP-4 was surprising. However, since we have now shown that the peptide doesn't enter cells, but instead appears to bind tightly to the cell membrane, the inability for DP-4 to kill tumor cells is consistent with the lack on internalization results.

Task 2. To ability of the p53-terminal peptides to induce endogenous p53 transcriptional activity will be examined by transfection of the breast cancer and non-breast cancer cells with a p53-dependent luciferase reporter followed by addition of increasing concentrations of the p53 peptide fusions. The level of luciferase will be measured 6 hours post-addition of the peptide. (Months 1-6, Year 2)

Progress: Work from our lab as well as work performed in the laboratories of several collaborators suggest that the effects of the c-terminal p53 peptide may not be sequence dependent, but instead may be based on a charge effect. Thus we are not pursuing the p53 c-terminal peptide as a therapeutic agent.

Task 3. The ability of the p53, NBD and Smac peptides to sensitize the tumor lines to the apoptotic effects of rTRAIL, etoposide and radiation will be examined. Increasing concentrations of the various peptide fusions will be added to cells followed by addition of suboptimal doses of rTRAIL and etoposide, as well as radiation. (Months 6-12, Year 2; Months 1-6, Year 3)

Progress: The results presented above demonstrate that we have been able to identify a tumor specific peptide, able to bind to the cell surface or in the membrane of tumors cells. Moreover, the peptide is able to home to several different xenografted tumors in nude mice, found only in tumor tissue, but not other tissues 3 hours post-injection. However, the peptide is not internalized in contrast to the cationic transduction peptides or to other tissue specific transduction peptides. These results suggest that this peptide could be useful for delivery of diagnostic therapeutic agents to certain tumor types. Thus we are examining the ability of the peptide to home to several different types of xenograft tumor lines as well as several murine tumor lines. The experiments will be performed first with subcutaneous tumors, but then with metastatic tumors.

In addition, we are developing approaches to see if the tumor targeting peptide can be used to deliver transduction peptides to the tumor. For these experiments several different approaches are being tested. The first is to link the tumor targeted peptide to a cationic PTD (10K) to see if the peptide can be internalized into tumor cells, first in cell culture, but then in xenograft tumor models. In addition, the tumor peptide will be linked to the cationic transduction domain via a protease (MMP-2 and MMP-9) cleavage site so that the cationic PTD will be released upon binding to the tumor membrane via cleavage by proteases overexpressed in tumor cells. Finally, a longer peptide will be generated carrying the tumor targeting peptide fused to a string of negatively charged amino acids, able to neutralize the positively charged cationic PTD. This will be fused to a strong general PTD via a protease sensitive cleavage site. The approach of using a negatively charged peptide fused to a cationic PTD via a protease cleavage site has been shown to give tumor specific transduction by Roger Tsein and colleagues. The addition of the tumor targeting peptide should enhance the concentration of the peptide at the tumor site, resulting in more cleaved peptide and more transduction. If tumor specific transduction can be achieved with any of the tumor targeting peptide-cationic peptide fusions, then the peptide will be extended to include a general apoptotic agent such as an anti-microbial peptide or the Smac peptide.

In addition to performing studies with recombinant TRAIL protein, we have constructed adenoviral vectors expressing human membrane bound TRAIL as well as a soluble TRAIL. Transduction of a variety of tumor cells including MCF-7 breast tumor cells resulted in an increase in apoptosis in both the soluble and membrane bound groups as determined by Annexin staining. The progress for this task was outlined in the previous progress report. We currently have two manuscripts submitted that described the use of Smac-PTD peptides for inducing tumor apoptosis, either alone or in combination with TRAIL. The abstracts for these two pending papers are presented below.

Mushiake, H., Hitchins, M. R., Mai, J. C., Ng, B., Spencer, J. V., Duke, J. E., Seol, D. W. and Robbins, P. D. Adenoviral-TRAIL and Smac34-8K act synergistically to induce apoptosis of low CAR-expressing human glioma cells. *Gene Therapy*, under revision.

Abstract: One promising therapy for gliomas involves the use of adenovirus (Ad) to deliver genes encoding pro-apoptotic agents such as Apo2L/tumor necrosis factor-related apoptosis inducing ligand (TRAIL), a protein shown to specifically induce apoptosis in cancer cells. However, gliomas can exhibit resistance to infection by adenovirus due to low surface expression of the adenovirus receptor, CAR. Recent evidence demonstrated that peptides containing protein transduction domains (PTDs) enhance adenovirus infectivity. To determine if PTDs can enhance adenoviral infection of gliomas, Ad.EGFP was incubated with a panel of PTDs prior to infection of glioma cells. Measurement of the mean fluorescent intensity and the percent EGFP positive cells revealed that the PTDs promoted viral entry, but in a cell-type dependent manner with an 8K peptide being the most effective. To circumvent possible resistance of certain gliomas to TRAIL-mediated apoptosis, Ad.stTRAIL was pre-incubated with a peptide based on the first 34 amino acids of Smac (Smac34-8K), which enhances TRAIL-mediated apoptosis by blocking pro-apoptotic proteins. Smac34-8K greatly improved Ad.stTRAIL-induced apoptosis in the TRAIL-resistant, low CAR expressing CCF-SSTG1 glioma cells. These results demonstrate that the combination of cell permeable peptides, fused to certain peptides, proteins or small molecules, with adenoviral vectors expressing apoptotic proteins, may be an effective strategy in enhancing tumor cell apoptosis.

Hitchens, M., Mai, J. C. and Robbins, P. D. Transduction of the first 34 residues of the Smac protein both enhance and induce apoptosis in human prostate cancer cell lines. *Mol. Can. Ther.*, under revision.

Abstract: Prostate cancer cells eventually become resistant to apoptotic induction and are thus difficult to eradicate. One approach to this problem is to introduce pro-apoptotic agents, such as peptides, into prostate cancer cells using protein transduction technology. In this report, we used peptides derived from the amino terminus of the pro-apoptotic protein Smac. PTD5-Smac7 and PTD5-Smac34 consisted of the protein transduction domain PTD5 linked to the first 7 or 34 amino acids of mature Smac. PTD5-Smac7 had no effect on the viability of DU145 cells. In contrast, PTD5-Smac34 both enhanced TRAIL- and ectoposide-mediated death, as well as induced apoptosis on its own. To optimize the pro-apoptotic activity of the Smac peptides, a panel of protein transduction domains, (PTD), was tested for their ability to transduce DU145 cells identify 8 lysines (8K) as the most efficient PTD for prostates tumors. Smac peptides were synthesized containing either the first 12 or 34 amino acids of mature Smac linked to 8K, termed Smac12-8K and Smac34-8K respectively. Smac34-8K induced and enhanced apoptosis through a caspase independent pathway whereas Smac12-8K had no effect in DU145 and PPC1 cells. In DU145-tumor bearing nude mice, intratumoral injection of Smac34-8K significantly decreased tumor volume as compared with 8K. Taken together, these experiments demonstrate that peptides based on the first 34 amino acids of mature Smac may be more effective than shorter Smac peptides in treating prostate cancer.

As outlined above, we have shown that fusion peptides carrying an optimal PTD with the amino terminal region of Smac were able to induce apoptosis of prostate tumor, breast and glioma cell lines. In addition, we have also demonstrated that the Smac fusion peptide is able to enhance apoptosis conferred by TRAIL. We have constructed an adenoviral vector carrying a chimeric trimeric TRAIL that we are testing for ability to induce apoptosis in tumor cell lines in culture and in vivo. We currently have a manuscript under revision at Gene Therapy describing the ability of adenoviral gene transfer of sTRAIL to induce wide spread tumor apoptosis in vivo following intra-tumoral delivery. Our current strategy for treating breast tumors is to use the Smac-12K fusion peptide in combination with Ad.sTRAIL gene transfer. The Smac-12K peptide is able to enhance the infectivity of the tumor cells as well as enhance the sensitivity of the tumor cells to TRAIL.

Kim, C.-Y., Jeong, M., Kim, B.-M., Kim, W.-B., Ko, J. P., Kim, M.-H., Kim, M., Kim, T.-H., Robbins, P. D., Billiar, T. R., and Seol, D.-W. Adenoviral delivery of the gene encoding secretable trimeric TRAIL suppresses tumor growth in vitro and in vivo via apoptosis induction. *Gene Ther.*, under revision

Abstract: Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is a type II transmembrane cytokine molecule. Soluble TRAIL has been shown to induce apoptosis in a wide variety of cancer cells in vitro and to suppress tumor growth specifically without damaging normal cells and tissues in vivo. In our previous report, we have demonstrated that an artificial gene encoding the polypeptide composed of the three functional elements (a secretion signal, a trimerization domain and an apoptosis-inducing moiety of TRAIL gene sequence) expresses and secretes highly apoptotic trimeric TRAIL into the culture supernatant. Here, as an approach to TRAIL-based cancer gene therapy, we developed an adenoviral vector delivering the gene that encodes our secretable trimeric TRAIL (stTRAIL). This adenovirus (Ad-stTRAIL) potently induced apoptosis in vitro in cancer cell lines such as HeLa, MDA-MB-231, A549, HCT116 and U-87MG. In an animal xenograft tumor model bearing a human glioma cell line U-87MG, intra-tumoral delivery of Ad-stTRAIL dramatically suppressed tumor growth without showing detectable adverse

side effects. Histological analysis revealed that Ad-stTRAIL suppresses tumor growth by inducing apoptotic cell death. Contrary to the known rapid clearance of systemically delivered TRAIL protein from the blood circulation, stTRAIL expressed by Ad-stTRAIL in tumor tissues persisted for more than 4 days. Our data reveal that a gene therapy using Ad-stTRAIL has a promising potential to treat human cancers including gliomas.

Reportable Outcomes.

We previously have screened a panel of arginine and lysine rich transduction peptides for transduction of DU145, LNCaP and PC3 in cell culture, demonstrating that the most effective peptide for transduction was a peptide containing 12 lysines or arginines. The intracellular location of these peptides and their ability to deliver the pro-apoptotic KLAK and SMAC34 peptide to breast tumor cell lines is currently being examined. We also have initiated experiments using a peptide phage display library to screen for peptides able to target tumor cells following systemic delivery. Using this approach, we have identified a phage encoding the peptide SVSVGMKPSRP that was highly enriched in a subcutaneous glioma in nude mice with over 90% of the isolated phage encoding the same SVSVGMKPSRP peptide. The identification of an enriched peptide from the glioma screen suggests that the screening protocol is appropriately designed. Interestingly, the identified peptide is identical to a peptide previously identified by an M13 peptide phage display screen for peptide able to transduce prostate tumors. Our analysis of the ability of the SVSVGMKPSRP peptide to transduce tumor cells in culture suggests that it binds tightly to the surface or within the tumor cell membrane, but is not internalized inside the cells. Interestingly, it seems to bind tightly to a variety of tumor cell types in culture including lung, brain, prostate and breast tumors. Furthermore, it appears as if the peptide is able to target specifically to xenografted tumors within 3 hours post-injection either i.v. or i.p. Given the ability of the peptide to target specifically to the tumor site, not being taken up by cells in the liver, we are testing different versions of a chimeric peptide carrying the tumor-targeted peptide fused to a cationic peptide. Finally, we have demonstrated previously that PTD fusion peptides based on the amino terminus of mature Smac, a protein demonstrated to enhance apoptosis by blocking IAP family members, are able to induce and enhance apoptosis. We have generated an adenoviral vector expressing a chimeric trimeric soluble TRAIL protein, able to induce apoptosis in vivo following intra-tumor injection. We have demonstrated that preincubation of the Ad.stTRAIL virus with a peptide based on the first 34 amino acids of Smac (Smac34-8K) greatly improved Ad.stTRAIL-induced apoptosis in the TRAIL-resistant, low CAR expressing tumor cells. The improvement in TRAIL mediated apoptosis was due to enhanced infection with the Ad.sTRAIL virus as well as enhanced sTRAIL mediated apoptosis. Thus we propose to use a combination of a PTD-Smac peptide with the Ad.sTRAIL virus for treatment of breast cancer, which will be tested in the next year.